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(54) Title: NON-A, NON-B HEPATITIS HEPATOCYTE CELL CULTURE

(57) Abstract

A Non-A, Non-B hepatitis (NANBH) viral in vitro cell culture is disclosed. Primary hepatocytes were isolated and cultured from a chimpanzee during the acute phase of an experimental NANBH virus infection. The differentiated hepatocyte cell culture was maintained in a serum-free medium comprising Williams medium E, a hepatocyte proliferogen, transferrin, serum albumin, corticosteroide prolactin, thyrotropin-releasing factor, cholera toxin and ethanolamine. The cultured hepatocytes tested positive for the expression of a NANBH-associated cytoplasmic antigen. The presence of this cytoplasmic marker suggested persistence of the infection in vitro. The production of infectious virus in vitro was confirmed by inoculating a chimpanzee with NANBH virus-infected tissue culture and later documenting the NANBH development in the chimpanzee.

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DESCRIPTION:

NON-A, NON-B HEPATITIS HEPATOCYTE CELL CULTURE

FIELD OF THE INVENTION

The present invention relates to an in vitro cell culture medium capable of maintaining in culture Non-A, Non-B hepatitis (NANBH) virus. More particularly, this invention relates to a serum-free primate hepatocyte cell culture medium which can maintain NANBH virus in culture.

BACKGROUND OF THE INVENTION

Non-A, Non-B hepatitis has long been recognized as a virus-induced disease, distinct from other forms of viral-associated liver diseases, including hepatitis A virus (HAV) and B virus (HBV), and the hepatitis induced by cytomegalovirus (CMV) or Epstein-Barr virus (EBV). Yet, despite years of extensive research, the NANBH virus has eluded isolation, characterization and in vitro cultivation. A considerable amount of data suggests the existence of two or more types of NANBH virus. The two general types are distinguished by mode of transmission, namely parenteral and enteric. Of the two, the parenterally transmitted form is associated with chronic hepatitis. Shorey, James, Amer. J. Med. Sci. 289:251-261 (1985).

A decade has passed since the first experimental transmission of the human infectious NANBH agent to a chimpanzee, the only reliable animal model for this disease. Yet, to date, no tissue culture system has been developed which would maintain NANBH virus in culture. Consequently, the limited availability of an animal model and the absence of an in vitro tissue culture model have severely hampered the isolation and characterization of this elusive agent. Without the ability to isolate and characterize the NANBH virus, researchers are stymied in their attempts to develop diagnostic reagents, therapeutic compounds, and vaccines for this disease. Due to the lack of a diagnostic tool or vaccine, approximately 90% of post-transfusion associated hepatitis can be

attributed to this putative viral agent. Some researchers have suggested that this infectious agent has properties consistent with the togavirus family. It is of interest that we have observed particles by electron microscopy in partially purified human serum containing infectious NANBH virus that have the morphology of the togavirus family.

To date, the inability to maintain differentiated primate hepatocyte cultures has probably been the biggest single obstacle to the isolation, characterization and in vitro culture of human infectious NANBH virus. While avian hepatocyte cell cultures capable of replicating duck hepatitis B have been reported (Tuttleman et al., J. Virol. 58:17 (1985)), these cell cultures have not been useful for the propagation of human hepadna virus. Hepadna viruses exhibit a narrow host range: chimpanzees are the only species other than man that can be infected with human hepatitis B virus. Recently, primary human hepatocytes maintained in a medium containing dimethyl-sulfoxide were shown to be susceptible to exogenous infection with HBV. These cultures are short-lived, poorly differentiated, and have not been shown to be susceptible to NANBH virus. Fourel et al., J. Virol. 62:4136-4143 (1988).

Recently, a hormonally defined, serum-free differentiated primate hepatocyte cell culture medium has been developed. See U.S.S.N. 222,569, filed July 20, 1988. Cultured differentiated primate liver cells offer many advantages for biochemical, viral culture and carcinogenesis studies. A system in which adult primate hepatocytes can be successfully cultured while maintaining differentiation of cell function and morphology offers tremendous possibilities in aiding the study of acute and chronic viral hepatitis and isolation of hepatotrophic viruses.

Thereis, therefore, a need for an in vitro NANBH viral cell culture medium. Such a medium which can sustain replication and propagation of NANBH virus may

ultimately lead to the isolation and characterization of the NANBH virus and eventually lead to diagnostic and therapeutic agents specific for combatting NANBH virus infection.

SUMMARY OF THE INVENTION

The present invention provides an in vitro cell culture of NANBH virus which includes NANBH-infected primate hepatocytes sustained in a serum-free medium comprising a basal cell culture medium, a hepatocyte proliferogen, serum albumin, a corticosteroid such as hydrocortisone, one or both of somatotropin or prolactin, a growth/releasing factor, cholera toxin and ethanolamine.

The present invention also provides a serum-free, cell-free isolate of NANBH virus. The isolate of NANBH virus is obtained as either a culture medium supernatant or a lysate of the in vitro cell cultured NANBH virus-infected hepatocytes.

Further, the present invention includes methods of producing NANBH virus infection in chimpanzees. Such controlled NANBH virus infection of chimpanzees should provide an experimental model for the study of NANBH virus infection and serve as reservoir for production of antibodies to NANBH virus. The NANBH virus infection is induced by inoculating chimpanzees with an infectious amount of an inoculum comprising an in vitro culture of NANBH virus-infected hepatocytes, a cell-free supernatant of the in vitro culture, a lysate of the in vitro culture, or cultured hepatocytes separated from their in vitro culture medium.

Further, the present invention provides a method of confirming NANBH viral infection in a host. The method involves excising hepatocytes from the host, culturing the hepatocytes, and observing cytopathic effects of the cultured hepatocytes after about two to four weeks. The cytopathic effects of the hepatocytes is indicative of NANBH virus infection.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following described serum-free media exemplifies the formulations of the present invention which are useful in sustaining NANBH virus in primate hepatocyte cell culture. While the examples demonstrate in vitro culturing of NANBH virus-infected chimpanzee hepatocytes, the culturing medium and techniques should be understood to apply as well to include in vitro culturing of NANBH virus in human hepatocytes.

In the described media of Table 1, Williams Medium E (WME) served as a basal medium. Although WME is presently preferred as the basal medium of the serum-free medium of the present invention, it will be understood by those skilled in the art who have the benefit of this disclosure that other commercial media formulations can be expected to give satisfactory results. For instance, a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (see Salas-Prato, in Growth of Cells in Hormonally Defined Media, Book A, G. H. Sato, et al., Eds., Cold Spring Harbor Laboratory, pp. 615-624 (1982)) or RPMI 1640 (Gibco) (see Enat, et al., Proc. Natl. Aca. Sci. USA 81:1411 (1984) and Sell, M. A., et al., "Long-term culture and passage of human fetal liver cells that synthesize albumin," In Vitro Cell. Dev. Biol. 21:216-220 (1985)) should give satisfactory results when supplemented with the supplements listed in Table 1.

TABLE 1

Supplement	Medium Concentration
EGF	100 ng/ml
Insulin	10 µg/ml
Glucagon	4 µg/ml
BSA	0.5 mg/ml
Linoleic Acid	5 µg/ml
Hydrocortisone	10^{-6} M
Selenium	10^{-7} M

Supplement	Medium Concentration
Cholera Toxin	2 ng/ml
LGF	20 ng/ml
Transferrin	5 µg/ml
Ethanolamine	10^{-6} M
Prolactin	100 ng/ml
Somatotropin	1 µg/ml
TRF	10^{-6} M

Although the exact function of many of the various additives with which the basal medium is supplemented is not well-defined, several of the supplements can be grouped in provisional categories to facilitate the description of the media formulation of the present invention. For instance, the term "hepatocyte proliferogen," as used herein, refers to one or more of the several growth factors or hormones, such as epidermal growth factor (EGF), insulin, liver growth factor (LGF), and glucagon, all of which have been implicated in controlling liver growth in vivo (Salas-Prato, supra, at 615).

The term "transport protein" as used herein refers to those proteins found in the serum which include as one of their functions the transport of certain substances in the blood. Such proteins include serum albumin, which may be advantageously used in the commonly available form of bovine serum albumin (BSA), and transferrin. However, liver cells synthesize transferrin such that satisfactory hepatocyte maintenance may be achieved without the addition of that transport protein.

The trace metal specifically contemplated for use in the medium of the present invention is selenium; however, WME contains copper, zinc, cobalt and iron and either WME, or other basal media, can be additionally supplemented with either or both of zinc and/or copper depending upon the original condition of the hepatocytes

and whether the basal medium includes either or both of those trace metal(s).

The literature reports the use of several growth and/or releasing factors which have been used for culturing liver cells, including thyrotropin-releasing factor (TRF), fibroblast growth factor, platelet-derived growth factor, multiplaction-stimulating activator, and endothelial cell growth supplement (ECGS) (for a review, see Leffert, H. L. and K. S. Koch, "Hepatocyte growth regulation by hormones in chemically defined media: A two-signal hypothesis," in Growth of Cells in Hormonally Defined Media, Book A, G. H. Sato, et al. (Eds.), Cold Spring Laboratory, pp. 597-613 (1982)). Any one or more of those growth/releasing factors can be added to the media of the present invention, depending upon factors such as the original condition of the hepatocytes and the particular protocol to be utilized.

Although the supplements are set out in specific proportions in the following table, it will be understood by those skilled in the art who have the benefit of this disclosure that those proportions can be, and in some circumstances, must be, varied. For instance, ECGS has been found not to be required for maintenance of the hepatocytes. The concentration of glucagon in the media can be reduced. Also, there is some interchangeability between certain of the supplements. For instance, the addition of soybean lipids may be substituted for linoleic acid. In addition, the quality of the hepatocytes obtained from different isolations may require the use of different hepatocyte proliferogens. The media of the present invention, therefore, includes a range of proportions of each of the supplements as shown in Table 2.

TABLE 2

Supplement	Range	Preferred Range
EGF	≥ 25 ng/ml	50-100 ng/ml
Insulin	≥ 2 µg/ml	5-10 µg/ml
Glucagon	≥ 0.5 µg/ml	0.5-10 µg/ml
BSA	≥ 0.2 mg/ml	0.5-2 mg/ml
Soybean lipids	0-20 µg/ml	0-20 µg/ml
Linoleic acid	0-5 µg/ml	0-5 µg/ml
Hydrocortisone	≥ 10^{-9} M	10^{-8} - 10^{-6} M
Selenium	≥ 10^{-9} M	3 x 10^{-8} - 10^{-7} M
Cholera toxin	0-5 ng/ml	0-2 ng/ml
LGF	0-50 ng/ml	0-20 ng/ml
ECGS	0-60 µg/ml	0-60 µg/ml
Transferrin	0-10 µg/ml	0-5 µg/ml
Ethanolamine	≥ 10^{-8} M	10^{-6} M
Prolactin	0-200 ng/ml	100 ng/ml
Somatotropin	0-5 µg/ml	1 µg/ml
TRF	0-10^{-6} M	0-10^{-6} M

It will be further understood that, with respect to the proportions of each of those supplements, when it is stated that a media formulation includes, for instance, 10^{-6} M TRF, the media includes about 10^{-6} M TRF.

The several studies that were performed to evaluate the ability of the media formulation of Example 1 of the present invention to support NANBH viral replication will now be described. The isolation of NANBH virus-infected hepatocytes from chimpanzees is described in Examples 2 and 4.

EXAMPLE 1

Serum-Free Medium Formulation

The serum-free media formulation utilized WME as a basal medium supplemented with 10 mM HEPES, pH 7.4, 2.75 mg/ml $NaHCO_3$, and 50 µg/ml gentamycin. To

prepare the media, the supplements were added in the following quantities to 500 ml of WME in a sterile plastic bottle:

5 ml	50 mg/ml BSA, 500 µg/ml Linoleic Acid
0.5 ml	5 mg/ml Insulin
0.5 ml	5 mg/ml Insulin, 5 mg/ml Transferrin, and 5 µg/ml Selenium (ITS)
50 µl	10^{-2} M Hydrocortisone
5 µl	200 µg/ml Cholera toxin
0.5 ml	100 µg/ml EGF
50 µl	10^{-2} M Ethanolamine
0.5 ml	1 mg/ml Somatotropin
50 µl	1 mg/ml Prolactin
0.5 ml	10^{-3} M Thyrotropin Releasing Hormone
50 µl	200 µg/ml LGF
1 ml	2.0 mg/ml Glucagon

WME was purchased with L-glutamine and without $NaHCO_3$ from Hazelton Research Products, Inc. (Denver, Penn.).

EXAMPLE 2

Chimpanzee Experimental NANBH Infection

In order to obtain NANBH virus-infected hepatocytes for in vitro experimentation, a parenteral NANBH virus infection was induced in chimpanzee PTTx7, a 14-year-old female, by inoculation with 5 ml of a 20-fold concentrate of acute phase plasma of unknown titer derived from a second passage of the Hutchinson strain of NANBH virus. Progression of the NANBH virus infection was monitored by ALT/AST enzyme fluctuations from weekly blood samples and by histopathologic examination of periodic liver needle punch biopsies. All biopsies were processed identically using conventional techniques. Immediately after harvesting, the liver biopsies were fixed for 1-3 hours in neutral buffered 3.7% formalin, processed manually according to standard procedures, embedded in paraffin, sectioned at 4 microns and stained with hematoxylin and eosin. All sections were examined histologically by the same board certified veterinary pathologist.

Since the onset of clinical hepatitis was significantly delayed, a second inoculation of 1.5 ml ($10^{2.5}$ CID_{50}) NANBH virus Hutchinson inoculum was administered on week 10 to assure infection. However, the appearance of elevated ALT on week 12 indicated that the second inoculum either exacerbated the primary infection or was not required. The ALT profile of the animal exhibited a rise above normal values from 12-19 weeks post-inoculation, and a second ALT elevation occurred on week 39.

Liver wedge survery was performed on week 14 at the onset of definitive ALT elevation. Microscopic examination of liver tissue taken at this time revealed occasional collections of lymphocytes and macrophages in hepatic triads and in focal parenchymal areas. There were no other changes indicating a significant inflammatory response. Although minimal inflammation was present, this finding could be representative of normal liver tissue. Hepatocytes were isolated on week 14 under the pretense that maximal virus replication would occur prior to or during this stage of the disease manifestation.

A liver punch biopsy taken after ALT elevations (week 19) revealed an increased number of lymphocytes in portal areas and in the parenchyma of the liver. Associated with the parenchymal lesions were necrotic hepatocytes. The hepatocytes around central vein areas were often lightly stained and granular with minimal swelling of the cytoplasm. All these changes described indicated minimal, lymphocytic, multifocal, viral hepatitis.

Development of In Vitro NANBH Virus-Infected Hepatocyte Cell Culture

Ketamine hydrochloride was used as the immobilizing and pre-anesthetic agent. Surgery was performed under general anesthesia with non-hepatotoxic sodium pentobarbital. A liver wedge of approximately 10 g was perfused using a modification of established protocols

(Maslansky, C. J. and G. M. Williams, In Vitro Models for Cancer Research, Vol. II: Carcinomas of the LIver and Pancreas, M. M. Weber and L. I. Sekely (Eds.), CRC Press: Boca Raton, Fla., pp. 43-60 (1985)). A two-step perfusion procedure was employed with all solutions maintained at 37°C throughout the perfusion procedure. The initial perfusion lasted 10 minutes using 1 liter of Ca^{++}, Mg^{++}-free Hanks Balanced salt solution supplemented with 10 mM HEPES (pH 7.4), 0.5 mM EGTA, and 100 µg/ml gentamycin sulfate. The next perfusion was for 20 minutes at approximately 60 ml/min. of Williams Medium E (WME) supplemented with 10 mM HEPES (pH 7.4), 100 µg/ml gentamycin sulfate, and 200 units/ml collagenase Type I (300 units/mg, Sigma). The liver capsule was then removed with fine forceps and hepatocytes were dislodged by gentle agitation in 100 ml of collagenase solution. The hepatocyte suspension was filtered through several layers of gauze pads into an equal volume of cold WME containing 5% fetal bovine serum (FBS), 10 mM HEPES (pH 7.4), and 100 µg/ml gentamycin sulfate. Hepatocytes were sedimented at 50 x g for 5 minutes and cell pellets were resuspended in WME 5% FBS. Sedimentation was repeated twice, pellets were resuspended in 10 ml WME 5% FBS, and viability and cell density were determined by trypan blue exclusion.

PRIMARIA plates (Falcon) were coated with rat tail collagen (Michalopoulos, G. and H. C. Pitot, "Primary culture of parenchymal liver collagen membranes," Exptl. Cell. Res. 94: 70 (1975)) for 6 minutes at room temperature, the excess collagen was removed, and plates were dried overnight under U.V. light. Viable cells were plated at a density of 3-4 x 10^6 cells/60 mm dish. Cell attachment occurred during a 3-hour incubation at 37°C, 10% CO_2 in WME 5% FBS, at which time cell monolayers were gently washed one time with WME and re-fed with serum-free medium formulation prepared as described in Example 1 above. The medium was changed

24 hours after isolation and at 48-hour intervals thereafter.

The cultured hepatocytes displayed a typical hepatocyte morphology as observed by phase-contract microscopy on day 5 of culture. This morphology was maintained until days 21-28 when the cultures exhibited a degenerative process.

NANBH Hepatocyte Cell Culture Characteristics

The synthesis and secretion of albumin, apolipoprotein A-I, and apolipoprotein E were monitored by immunoblotting of aliquots of tissue culture medium. Briefly, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE,) and were electrophoretically transferred to Nylon-X nitrocellulose filters (Fisher) at 100 mA for 16 hours at 4°C. Unoccupied binding sites were blocked in 10% nonfat dry milk in phospate buffered saline (PBS) for 2 hours at 37°C. Membranes were incubated for 2 hours at 37°C in PBS-milk-Tween (PBS containing 5% nonfat dry milk, 0.3% Tween-20), using primary antibodies directed against human apolipoproteins A-I and E. Membranes were washed three times with PBS-Tween and incubated 1 hour at 37°C in PBS-milk-Tween with antibodies directed against each of the primary antibodies. Membranes were washed three times with PBS-Tween and incubated 1 hour at 37°C in PBS-milk-Tween with [^{125}I] protein A (8.5 µCi/µg, NEN). Membranes were washed three times with PBS-Tween and air dried. Immunoblots were autoradiographed at -85°C on XAR-5 film (Kodak) with intensifying screens.

The levels of apolipoproteins A-I and E increased in the cultures up to day 13, remained constant from day 13-28, and declined from day 28-45.

Albumin detected by this immunoblot procedure remained at constant levels throughout the culture period. Although albumin is a marker for differentiated hepatocytes, it is not as stringent of a marker for the differentiated state as is lipoprotein synthesis.

The decline in lipoprotein synthesis after 28 days in culture paralleled a degeneration in the hepatocyte cultures. The degeneration of primary hepatocytes after 3-4 weeks of culture was evident in cultures derived from two different NANBH-infected chimpanzees, but was not observed in cultures from a normal chimpanzee or chimpanzees with HBV infections. Normal hepatocyte cultures generally survive more than 100 days in the serum-free media. Further experimentation will be required to determine whether the degenerative process is due to viral-induced cytopathic effect.

To further characterize the differentiated state of the hepatocytes in vitro, the de novo synthesis of liver specific plasma proteins was analyzed. On day 17, cultures were labeled for 24 hours with [^{35}S]methionine. Plasma proteins were immune precipitated from the labeled medium and analyzed by SDS-PAGE.

Cultures were incubated in 2.5 ml of the serum-free media of Example 1 supplemented with 250 µCi [^{35}S]methionine (>800 Ci/mmol, ICN) for 24 hours. Medium was filtered and mixed with 1/10 volume of 10x CHAPS extraction buffer [final concentration 1.0% CHAPS (CalBiochem), 0.25 mM phenylmethyl sulfonyl fluoride, 10 mM EDTA, 0.05 M Tris (pH 8.0), 0.1 M NaCl, 100 µM leupeptin] and incubated for 1 hour at 4°C with agitation. Commercially obtained antibodies (CalBiochem, Boehringer Mannheim) directed against human plasma proteins (20 µl) were bound to protein A-agarose beads (50 µl, Repligen) for 1 hour in CHAPS extraction buffer on ice. The beads were washed two times with detergent wash buffer [CHAPS extraction buffer plus 1% deoxycholic acid and 0.1% SDS] and were incubated with the labeled medium overnight at 4°C with agitation. The beads were pelleted and washed three times with detergent wash buffer. Bound proteins were eluted with 50 µl electrophoresis sample buffer containing 2% SDS and 2% 2-mercaptoethanol, heated at 100°C for 10 minutes and analyzed by SDS-PAGE. Gels were processed for

fluorography with Autofluor (National Diagnostics), dried, and autoradiographed at -85°C on XAR-5 film.

This analysis suggested that the amount of plasma proteins synthesized in vitro reflected the concentrations found in plasma. The intensities of the polypeptide bands in descending order were albumin, alpha 1 antitrypsin, fibrinogen, transferrin, apo A-I and E, beta 2 microglobulin, pre-albumin, apo A-II and A-III, complements C3, C4 and C5, C-reactive protein, and apo C-2 and C-3. All markers examined were detected with the exception of alpha fetoprotein, which is a marker for poorly differentiated fetal or malignant liver tissue. The expression of numerous plasma proteins indicated that differentiated hepatocytes of parenchymal origin were maintained in culture.

Hepatocyte cultures grown on coverslips were analyzed at various times during the culture period for the presence of a novel NANBH virus-associated antigen that can be detected by immunocytochemical staining (Burk et al., "Detection of non-A, non-B hepatitis antigen by immunocytochemical staining," Proc. Natl. Acad. Sci. U.S.A. 81:3195-3199 (1984)). Typical cytoplasmic staining was observed in all samples examined with a tendency for the percentage of cells expressing this marker to increase with time in culture. However, the number of cells with definitive staining never increased above 10%.

The active replication of the virus in tissue culture was suggested by the presence of a NANBH virus-associated cytoplasmic antigen. In addition, the degeneration of the primary chimpanzee hepatocytes after 4 weeks of culture may have been due to the replication of the virus. Based on these findings, the production of infectious NANBH virus in the hepatocyte cultures was assayed by inoculation of a chimpanzee with tissue culture medium and monitoring the animal for disease manifestation. Previous experimentation with HBV suggested that the limited number of cells infected in

cultures resulted in lower viral titers than those observed in vivo. In addition, it was unknown whether the expression of the NANBH agent was transient during the culture period. Therefore, media samples from each time point (days 3-31) were pooled and concentrated eight-fold by ultrafiltration and used to inoculate an HBV immune, NANBH virus non-immune chimpanzee (PTTx196).

EXAMPLE 3

Tissue Culture-Derived NANBH Virus Inoculum

Tissue culture medium as described in Example 2 was collected at two-day intervals and passed through 0.45 µm filters and stored at -100°C. Equal amounts of each sample, days 3 through 31, were collected (190 ml total) and concentrated by pressure dialysis under N_2 gas at 4°C with an exclusion membrane of 30,000 MW (YM30, Amicon). The eight-fold concentrate (22 ml) was stored at -100°C until use as exemplified by Example 4.

EXAMPLE 4

Induction of In Vivo NANBH Virus Infection Using NANBH Hepatocyte Cell Culture Medium

Without a definitive probe to monitor NANBH viral expression in the medium of these hepatocyte cultures, it was necessary to obtain conclusive evidence for the active replication of NANBH virus by the induction of hepatitis in a chimpanzee with medium derived from the virus-infected cultures.

PTTx196, a 12-year-old male chimpanzee, received 10 ml of an eight-fold concentrate of tissue culture medium (Example 3) derived from hepatocyte cultures isolated during the acute phase of the experimental NANBH virus infection of PTTx7. A second inoculum of the same material (7 ml) was administered 12 weeks later.

Weekly blood samples and periodic liver needle punch biopsies were taken for analysis. A slight increase in ALT occurred on week 4 and microscopic examination of a liver punch biopsy at this time revealed minimal foci of

hepatocellular necrosis with two or three neutrophils associated with the necrosis. This represents a minimal change that can be occasionally observed in normal tissue but was of interest under these conditions. An ALT/AST inversion occurred on week 8 and a second liver needle punch biopsy taken at this time exhibited essentially normal tissue with no microscopic lesions recognized. Similar findings of normal tissue were observed in biopsy material taken on week 12.

Due to the delay in onset of clinical hepatitis, a second injection of the same inoculum (7 ml) was administered on week 12. This was followed by an elevation in ALT values beginning 3 weeks later. A persistent ALT elevation was observed 16-24 weeks after the first inoculation. The long incubation period may reflect the low titer of our initial inoculum. However, microscopic examination of a liver punch biopsy taken on week 14 exhibited signs of hepatitis. Foci of inflammatory cell accumulation were present in the hepatic parenchyma. Occasionally there were necrotic hepatocytes (Councilman bodies) associated with the inflammation. Kupffer cell hyperplasia was evident throughout the liver. There was also hydropic degeneration of hepatocytes in central vein areas. These changes were minimal but similar to those seen in the biopsy taken on week 4. Examination of liver punch biopsy material taken on week 17, during the period of elevated serum ALT, indicated acute hepatitis characterized by hydropic degeneration with loss of hepatocytes in centrilobular areas.

For electron microscopy, the liver biopsy was fixed with cold 3% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4) and postfixed for 1 hour at 4°C in 1% osmium tetroxide. Dehydration in ethanol and propylene oxide was followed by embedding in Epon 812. Sections were cut with a diamond knife on an LKB UM I ultramicrotome, stained with saturated aqueous uranyl acetate and lead citrate, and examined with an AEI EM6B electron

microscope. Magnification scales were calibrated using a carbon grating replica (54,800 lines/inch); E. F. Fullam Inc., Schenectady, N.Y.).

Electron microscopy performed on a liver biopsy taken on week 17 revealed the presence of convoluted tubules in the cytoplasm of the hepatocytes. The presence of these tubules has been used as a diagnostic marker for NANBH in chimpanzees. These results further substantiate that the clinical disease was due to inoculation with NANBH virus, derived from the tissue culture medium.

Plasma samples taken from PTTx196 on weeks 0, 16 and 22 of this experimental NANBH infection were analyzed for seroconversion in response to CMV, EBV, HBV, HSV and spumavirus. These agents may cause hepatitis or be transmitted by this methodology. No increase in antibody titer was observed by specific assay for CMV, EBV, HBV, spumavirus, and HSV. These results confirm that the disease transmitted to PTTx196 was caused by an NANBH agent.

Without a definitive probe to monitor viral expression in the medium of these hepatocyte cultures, it was necessary to obtain conclusive evidence for the active replication of NANBH virus by the induction of hepatitis in a chimpanzee with medium derived from the infected cultures. The possibility that the infectious virus detected in the tissue culture medium was residual virus present in the hepatocytes at the time of isolation is extremely remote. Extensive washing occurred during the perfusion/collagenase procedure (2 liters) and the pelleting and resuspension (4 times) of the hepatocytes prior to plating. In addition, by day 3 in culture four changes of medium had been performed. Thus, this experiment documents the feasibility of culturing hepatocytes isolated during the acute stages of an experimental NANBH virus infection. This system should prove beneficial for identifying and characterizing the NANBH agent, leading to the elucidation of its mechanism of replication and persistence in chronic infection.

Those skilled in the art who have the benefit of this disclosure will recognize that changes in the formulation of the serum-free medium of the present invention can be made without compromising the ability of the media to support the long-term culture of NANBH virus-infected primary hepatocytes. All such changes are considered to be within the spirit and scope of the present invention as defined by the following claims.

CLAIMS

What is claimed is:

1. An in vitro culture of NANBH virus comprising:
NANBH virus-infected primate hepatocytes,
a basal cell culture medium,
a hepatocyte proliferogen,
serum albumin,
a corticosteroid,
one or both of somatotropin or prolactin,
a growth/releasing factor,
cholera toxin and
ethanolamine.

2. The in vitro culture of claim 1 wherein the basal cell culture medium is Williams Medium E, Dulbecco's modified Eagle's medium, or Ham's F12 medium.

3. The in vitro culture of claim 1 wherein said hepatocyte proliferogen is one or more of insulin, glucagon, liver growth factor, or epidermal growth factor.

4. The in vitro culture of claim 1 additionally comprising a trace metal.

5. The in vitro culture of claim 4 wherein said trace metal is selenium, zinc or copper.

6. The in vitro culture of claim 4 wherein said trace metal is selenium.

7. The in vitro culture of claim 1 additionally comprising transferrin.

8. The in vitro culture of claim 1 additionally comprising linoleic acid.

9. The in vitro culture of claim 1 wherein the growth/releasing factor is one or more of thyrotropin-releasing factor, fibroblast growth factor, platelet-derived growth factor, multiplication stimulating actuator or endothelial cell growth supplement.

10. The in vitro culture of claim 1 wherein the growth/releasing factor is thyrotropin-releasing factor.

11. The in vitro culture of claim 1 wherein the primate hepatocytes are chimpanzee hepatocytes.

12. The in vitro culture of claim 1 wherein the primate hepatocytes are human hepatocytes.

13. The in vitro culture of claim 1 wherein the NANBH virus is a parenterally transmitted NANBH virus.

14. An in vitro culture of NANBH virus culture comprising:

NANBH virus-infected chimpanzee hepatocytes,	10^6-10^7 cells/60 mm tissue culture dish
Epidermal growth factor	25-100 ng/ml,
Insulin	2-10 µg/ml
Glucagon	0.5-10 µg/ml,
Bovine serum albumin	0.2-2 mg/ml,
Linoleic acid	0-5 µg/ml,
Hydrocortisone	10^{-9} - 10^{-6} M,
Selenium	10^{-9} - 10^{-7} M,
Cholera toxin	1-5 ng/ml,
Liver growth factor	0-50 ng/ml,
Transferrin	0-10 µg/ml,
Ethanolamine	10^{-8} - 10^{-6} M,
Prolactin	0-200 ng/ml,
Somatotropin	0-5 µg/ml,
Thyrotropin-releasing factor	0-10^{-6} M

15. The in vitro culture of claim 14 wherein the NANBH virus is a parenterally transmitted NANBH virus.

16. A viral composition comprising:
serum-free, cell-free NANBH virus.

17. The viral composition of claim 16 wherein the serum-free, cell-free NANBH virus is a supernatant of an in vitro culture of NANBH virus according to claim 1.

18. The viral composition of claim 16 wherein the serum-free, cell-free NANBH virus is a lysate of an in vitro culture of NANBH virus according to claim 1.

19. The viral composition of claim 16 wherein the NANBH virus is a parenterally transmitted NANBH virus.

20. A method of producing NANBH virus infection in a non-immune chimpanzee comprising:
inoculating the non-immune chimpanzee with an infectious amount of an inoculum comprising an in vitro culture of NANBH virus according to claim 1.

21. The method of claim 20 wherein the inoculum comprises a lysate of an in vitro culture of NANBH virus according to claim 1.

22. The method of claim 20 wherein the inoculum comprises a cell-free supernatant of an in vitro culture of NANBH virus according to claim 1.

23. The method of claim 20 wherein the inoculum comprises a serum-free, cell-free NANBH virus.

24. The method of claim 20 wherein the inoculum comprises NANBH virus-infected primate hepatocytes cultured in vitro according to claim 1.

25. The method of claim 20 wherein the NANBH virus is a parenterally transmitted NANBH virus.

26. A method of confirming NANBH viral infection in a host comprising:

excising hepatocytes from the host,

culturing the hepatocytes in an in vitro culture medium according to claim 1, and

after about 2 to about 4 weeks, observing cytopathic effects of the cultured hepatocytes, said cytopathic effects indicative of NANBH virus infection.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00915

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 7/00; C12Q 1/70, 1/02; A01N 63/00
U.S. CL: 435/235, 5, 29; 424/93

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S. CL:	435/235,5,29,240.31,948;424/93

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *

DATA BASES: CHEMICAL ABSTRACTS (CAS) 1967-1990; BIOLOGICAL ABSTRACTS (BIOSIS) 1969-1990. SEE ATTACHMENT FOR SEARCH TERMS.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	Biological Abstracts, Volume 733, Issued 1981, BURK et al, "Ultrastructural changes and Virus-like particles localized in Liver Hepatocytes of CHIMPANZEES infected with Non-A Non-B Hepatitus", Abstract # 18396. See entire Abstract .	1-6,8-13 20-26
Y	The Journal of Biological Chemistry, Volume 261, No.8, Issued 15 March 1986, Edge et al, "Cultured human hepatocyles" pages 3800-3806. See entire Article.	1-6,8-13 20-26
Y	In Vitro Cellular and Developmental Biology Volume 24 No. 3, March 1988, Salas-Prato et al, "Attachment and multiplication, Morphology and protein production of human fetal primary liver cells cultured in hormonally defined media", pages 230-238. See entire Article.	1-6,8-13, 20-26
Y	Biochemical Journal, Volume 243, issued 1987, Diaz-Gil er al," Identification of a liver growth factor as an albuminbilirubin complex", pages 443-448. See entire Article	14-15
X / Y	US, A 4,464,474 (COURSAGET et al) 07 AUGUST 1984 See entire document.	16,19 / 17-18

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 MAY 1990	12 JUN 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	GAIL E. POULOS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	In Vitro, Volume 18, No. 1, Issued January 1982, Marceau et al,"Growth and Functional Activities of neonatal and adult rat hepatocyles cultured on fibronectin coated substratum in serum-free medium" Pages 1-11, See entire article.	7-14
Y	In Vitro cellular and Developmental Biology, Volume 25, No. 2, Issued February 1989, Landford et al, "Analysis of plasma protein and lipo protein synthesis in long-term primary cultures of baboon hepatocytes maintained in serum-free medium" pages 174-182. See entire article.	1-6,8-13. 20-26

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ATTACHMENT to Form PCT/ISA/210 Part II

FIELDS SEARCHED

SEARCH TERMS

Hepatocytes
Serum free
Linoleic
Liver growth factor
EGF
Prolactin
Non A nom B hepatitis Virus
J.R. Jacob
R.E. Landford
K.H. Burk